BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Molecular Immunology, Vol. 22, pp. 145–154, 1985
Printed in Great Britain

ANTIBODIES ELICITED BY INFLUENZA VIRUS HEMAGGLUTININ FAIL TO BIND TO SYNTHETIC PEPTIDES REPRESENTING PUTATIVE ANTIGENIC SITES*

ANN NESTOROWICZ,† GEOFFREY W. TREGEAR,‡ CHRISTINA N. SOUTHWELL,‡
JOHN MARTYN,† JULIE M. MURRAY,† DAVID O. WHITE† and DAVID C. JACKSON†
†Department of Microbiology, University of Melbourne, Parkville, Victoria 3052, Australia; and
‡Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria 3052, Australia

(*First received* 4 *July* 1984; *accepted in revised form* 20 *August* 1984)

Abstract—A number of peptides of the hemagglutinin (HA) of X-31 influenza virus have been synthesised. The amino acid sequences of some of these peptides represent regions of HA which have been postulated [Wiley *et al.*, *Nature, Lond.* 289, 373–378 (1981)] to form the antigenic sites of this molecule. Animals were immunized with free peptide or peptide conjugated to a carrier and the resulting antisera examined for their capacities to bind to homologous peptide, whole HA, reduced and alkylated HA, and intact virus. Not all peptides examined in this way were immunogenic. Only antibodies raised against the *C*-terminus of HA_1 peptide displayed binding to virus. This antiserum bound to the intact HA but not to the reduced and alkylated form of the molecule. These results raise questions as to the feasibility of using synthetic peptides of the influenza HA in short linear sequences to elicit neutralising antibody.

INTRODUCTION

Antigenic analysis of viral proteins has received a great deal of attention with numerous publications [e.g. Learner *et al.* (1981), Baron and Baltimore (1982), Bittle *et al.* (1982), Green *et al.* (1982), Emini *et al.* (1983), Grandgenett *et al.* (1983) and Tamura *et al.* (1983)] reporting that chemically synthesised short peptides are capable of eliciting antibodies that react with the native protein. An obvious consequence of such findings is the possibility of constructing vaccines composed of those synthetic peptides which are found to elicit antibodies capable of neutralizing viral infectivity.

The feasibility of this approach has been reported using a number of viruses. Langbeheim *et al.* (1976) showed that a 20 amino acid residue synthetic peptide representing part of the coat protein of MS-2 coliphage induced antibodies capable of neutralising the virus. Subsequently, the antigenic structure of other viruses such as tobacco mosaic virus (Altschuh *et al.*, 1983), turnip yellow mosaic virus (Quesniaux *et al.*, 1983), hepatitis B virus (Prince *et al.*, 1982), influenza virus (Jackson *et al.*, 1982; Müller *et al.*, 1982; Green *et al.*, 1981) and immunochemical (Jackson *et al.*, 1982*b*; Wiley *et al.*, 1981) structures of influenza virus 1982; Rowlands *et al.*, 1983) and poliovirus (Emini *et al.*, 1983) have been studied using the synthetic peptide approach. Only in the cases of MS2 coliphage (Langbeheim *et al.*, 1976), foot and mouth disease virus (Bittle *et al.*, 1983) and poliovirus (Emini *et al.*, 1983) have neutralising antibodies been obtained using synthetic peptides.

As detailed information about the primary [for review see Ward (1981)], three-dimensional (Wilson *et al.*, 1981) and immunochemical (Jackson *et al.*, 1982*b*; Wiley *et al.*, 1981) structures of influenza virus hemagglutinin (HA) is available we have examined a number of synthetic peptides of this molecule. HA is the major surface glycoprotein of the influenza virus and is the protein against which neutralising antibodies are directed. The HA appears as "spikes", composed of three identical subunits, which project from the viral envelope. Each monomer is comprised of two disulphide-linked polypeptide chains designated HA_1 and HA_2 (Laver, 1971).

On the basis of the finding that amino acid substitutions of variant viruses selected by monoclonal antibodies cluster into four regions on the three-dimensional model of HA, Wiley *et al.* (1981) have proposed that the molecule possesses four antibody-binding sites. These conclusions have been supported by the findings of others (Webster and Laver, 1980; Gerhard *et al.*, 1981; Breschkin *et al.*, 1981; Jackson *et al.*, 1982*b*). These antigenic sites, here designated "loop", "tip"/"interface" and "hinge" [Fig. 1, model after Wiley *et al.* (1981)], are located on the HA_1 polypeptide.

We now report the synthesis of a number of peptides of the HA of X-31 influenza virus and an evaluation of their antigenic and immunogenic properties. Four of these peptides comprising the loop (residues 140–150), tip (181–204) and hinge (46–55

*This work was supported by a grant from the National Health and Medical Research Council of Australia.

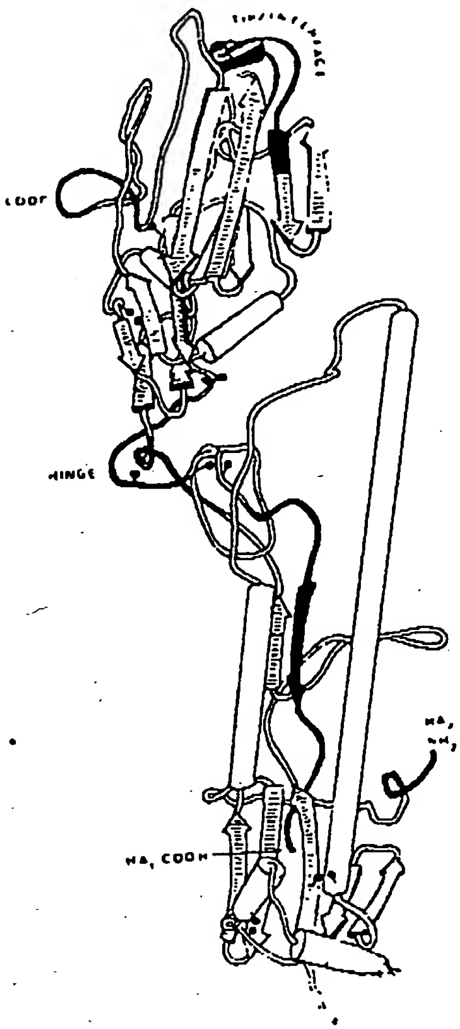

Fig. 1. Diagram of the monomer subunit of the HA of X-31 virus showing the locations of the putative antibody-binding sites, "loop", "hinge" and "tip/interface". Modified from Wiley *et al.* (1981). The approximate locations of the synthetic peptides used in this study within the intact molecule are shown by solid lines.

and 272–280) encompass regions of the molecule which form three of the antibody-binding sites proposed by Wiley *et al.* (1981). In contrast, the other two peptides are derived from regions of the HA which have a highly conserved amino acid sequence. One of the latter, the "fusion peptide" (residues 1–13 in HA_2), is extremely hydrophobic and evidence suggests that this region is directly involved in the fusion process between viral and cellular membranes (Huang *et al.*, 1981; White *et al.*, 1981; Skehel and Waterfield, 1975; Gething *et al.*, 1978; Richardson *et al.*, 1980; Skehel *et al.*, 1982). The other peptide (residues 305–328) contains the carboxy-terminus of HA_1 which is located in the stem region of the molecule.

MATERIALS AND METHODS

Synthesis of peptides

The peptides listed in Table 1 were prepared by the solid-phase procedure using a Beckman model 990 synthesizer. The general synthetic protocols used were based on the methods developed by Merrifield and Kent [see, for example, Barany and Merrifield (1980) and Kent (1980)] and are described in detail elsewhere (Tregear *et al.*, in preparation).

Peptides were assembled in a stepwise fashion on a benzhydrylamine, 1% cross-linked polystyrene resin (Protein Research Foundation, Osaka, Japan) with *N*-tertiarybutyloxycarbonyl amino acid derivatives. Our strategy was to prepare the peptides with the α-amino and α-carboxyl terminal groups blocked. For peptides containing cysteine, the free sulfhydryl groups were protected (with either acetamidomethyl or ethylmercapto groups to prevent dimerization. Following assembly of the complete sequence the peptide resin was treated with acetic anhydride in dimethylformamide to acetylate the amino terminus. Cleavage of the peptide from the resin with anhydrous hydrogen fluoride at 0°C for 30 min in the presence of anisole (10%) gave the crude peptide acetylated at the amino terminus and with the carboxyl terminus as the amide derivative. In the case of the HA_1 *C*-terminus peptide (305–328) synthesis was performed on a Thr-OCH_2-phenylacetamidomethyl polystyrene resin according to the procedures described by Kent (1980). Acetylation was not carried out at the final step of the peptide assembly and hydrogen fluoride cleavage gave the (305–328) peptide with free terminal amino and carboxyl groups.

The crude peptides were purified by gel chromatography using BioGel P6 or TSK-40 columns in 1 *M* acetic acid or 1 *M* ammonium bicarbonate. Further purification was effected by ion-exchange chromatography on CM- or DE-cellulose, or, where appropriate, peptides were collected from C-18 reverse-phase HPLC columns. The synthetic peptides were assessed for homogeneity by amino acid analysis and analytical HPLC.

Conjugation of peptides

Synthetic peptides were conjugated to KLH using 1-ethyl-(3-dimethyl-amino propyl) carbodiimide (Sigma Chemical Co.) as described by Goodfriend *et al.* (1964). Typical conjugation ratios were 200–500 moles of peptide per mole of KLH.

Viruses

The influenza viruses used were: A/PR/8/34-(H1N1), A/Vic/112/76(H3N2), A/Texas/1/77(H3N2), A/Vic/186/82(H3N2) and the recombinants, X-31, bearing the HA of A/Aichi/68(H3N2) and derived by reassortment between A/Aichi/68 and A/PR/8/34-

Table 1 Amino acid sequences of synthetic peptides of X-31 HA*

		Mol wt
HA_1		
Tip peptide	^{321}C V I I H P S T N Q E Q T S L Y V Q A S G R R V T V^{344}	2595
Loop peptide	^{134}K R G P G S G F F S R^{146}	1195
Hinge peptide A	^{46}S S T C K I C N N P^{55}	1019
Hinge peptide B	^{272}A P I D T C I S E^{280}	947
Hinge peptide A-B	^{46}S S T C K I C N N P^{55} (S, S) ^{272}A P I D T C I S E^{280}	1965
C-terminus HA_1 peptide	^{302}C P K Y V K Q N T L K L A T G M R N V P E K Q T^{329}	2748
HA_2		
Fusion peptide	^{1}G L F G A I A G F I E N G^{11}	1265

*The numbering of the single-letter code amino acid sequences is that used by Ward (1981). With the exception of HA_1(305-328) each of the peptides has its carboxyl terminal in the amide form and the amino terminal blocked as the *N*-acetyl derivative. The cysteines in hinge peptide A and hinge peptide B are the ethylmercapto derivatives. Both these peptides have been linked by the normal cysteine disulfide bond in hinge peptide A-B.

(H1N1). Shearwater$_H$-Bel$_N$, bearing the HA of A/Shearwater/E. Aust./172(H6) and the neuraminidase of A/Bellamy/42(N1), and Port Chalmers$_H$-Bel$_N$ bearing the HA of A/Port Chalmers/1/73(H3) and neuraminidase of A/Bellamy/42(N1). Viruses were grown in 10-day embryonated chicken eggs and purified by sucrose gradient centrifugation after adsorption to and elution from chicken erythrocytes (Laver, 1964).

HA

HA was isolated by electrophoresis of purified disrupted virus on cellulose acetate blocks (Laver, 1964). Reduction and carboxymethylation of HA was carried out as previously described (Jackson *et al.*, 1978). HA was also prepared by digestion of virus with bromelain (Sigma Chemical Co.) according to Brand and Skehel (1972). Briefly, virus was digested at 37°C for 18 hr in 0.1 *M* Tris-HCl, pH 8.0, 0.05 *M* 2-mercaptoethanol at an enzyme:virus protein ratio of 1:1. Bromelain HA was then purified by density centrifugation over a 5–20% sucrose gradient in phosphate-buffered saline (PBS).

Antisera

Antisera were raised by intramuscular inoculation of antigen (100 μg), emulsified in Freund's complete adjuvant, into outbred New Zealand rabbits at monthly intervals over a period of 6 months. Rabbits were bled 7–10 days after each boost and antisera monitored for antibody.

Antisera obtained from animals inoculated with virus were absorbed with Shearwater$_H$-Bel$_N$ virus to remove antibodies directed aginst the carbohydrate antigen according to previously described methods (Jackson *et al.*, 1978, 1981).

Immunoglobulin G (IgG) was prepared from rabbit serum by affinity chromatography on protein A–Sepharose CL-4B (Pharmacia).

Monoclonal antibodies H14A2, H14A20 and H14A21 were a generous gift from Dr Walter Gerhard.

Radioiodination

Bromelain HA, synthetic peptides and protein A were iodinated using either the Bolton and Hunter reagent (Bolton and Hunter, 1973) or a modification (Jackson, 1980) of the chloramine-T method (Greenwood *et al.*, 1963).

Radioimmunoassays (RIAs)

A number of different RIAs were employed in this study. A solid-phase assay (Brown *et al.*, 1980) was used in which antigen was dried onto wells of a polyvinyl microtitre tray by incubation at 37°C for 3 hr. After treating with a solution of bovine serum albumin to coat unoccupied areas of plastic, dilutions of IgG were added to the wells and incubated overnight at room temp. Binding of antibody was detected by the addition of radioiodinated protein A.

A solution-phase RIA (Jackson *et al.*, 1979*a*) employing radiolabeled synthetic peptides was also used. Briefly, dilutions of IgG were added to centrifuge tubes containing a constant amount of radioiodinated peptide. Following incubation at room temp for 3 hr, antigen–antibody complexes were isolated by the addition of a 20% (v/v) suspension of either protein A–Separose CL-4B or protein A–Sepharose saturated with rabbit antibody directed against mouse IgG. After a further 30-min incubation the immunoadsorbents were washed and the amount of radioactivity associated with each determined.

Finally, a modification of the solution-phase RIA was used in which unlabeled peptides were examined for their capacity to inhibit the binding of antibody to bromelain HA. Dilutions of peptide were incubated with constant amounts of IgG and radioiodinated HA. The amount of IgG used was that which bound 50% of the radiolabeled HA in the absence of inhibitor. Following incubation, antigen–antibody complexes were isolated and detected as described earlier.

Hemagglutination inhibition (HI) assays

Hemagglutination and HI assays were performed as described by Fazekas de St. Groth and Webster (1966).

RESULTS

Solid-phase RIAs

IgG isolated from various antisera raised against X-31 virus, synthetic peptides and synthetic peptide conjugates were examined in the solid-phase RIA for their abilities to bind to whole HA, HA which had been reduced and alkylated, and synthetic peptides. Purified immunoglobulin from 27 antisera were tested in this assay. In those cases where two or more animals were immunized with the same immunogen resulting in similar results, data obtained from a single serum only are presented. Where animals responded differently to immunisation both results are presented.

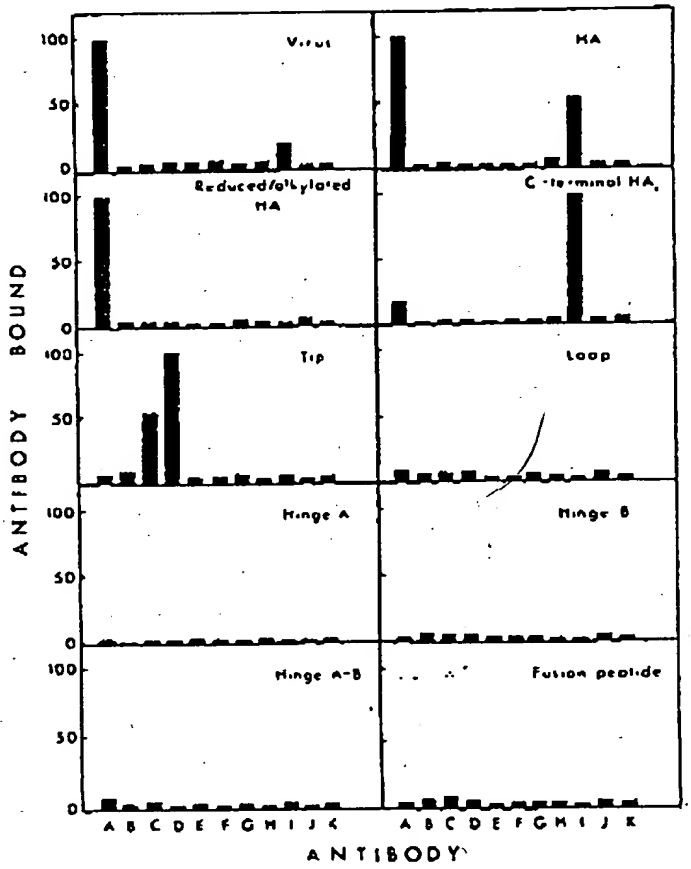

Fig. 2. Binding of antisera raised against X-31 virus, free synthetic peptides and synthetic peptide conjugates to X-31 virus, HA, reduced and alkylated HA, and synthetic peptides in a solid-phase RIA. Immunoglobulin G was purified from: (A) anti-(X-31 virus) serum, (B) anti-(loop peptide) serum, (C) anti-(tip peptide) serum, (D) anti-(tip–KLH conjugate) serum, (E) anti-(hinge A–B peptide) serum, (F) anti-(hinge A–B–KLH conjugate) serum, (G) anti-(fusion peptide) serum, (H) anti-(fusion peptide–KLH conjugate) serum, (I) anti-(C-terminal HA_1 peptide) serum, and (J) and (K) normal rabbit serum, and titrated against each antigen in a solid-phase RIA. The results shown are normalised values derived by expressing the level of binding obtained as a percentage of that obtained with a 1/10 dilution of anti-(X-31) IgG to virus.

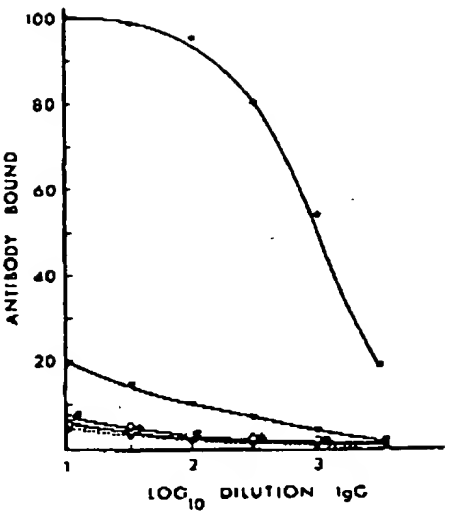

Fig. 3. Binding of anti-peptide and anti-(X-31 virus) IgG preparations to X-31 virus in solid-phase RIA. Dilutions of anti-(X-31 virus) IgG (●——●), anti-(*C*-terminal HA_1 peptide) IgG (■——■), anti-(tip peptide) IgG (◆——◆), anti-(fusion peptide) IgG (○——○), anti-(hinge A–B peptide) IgG (△——△), anti-(loop peptide) IgG (□——□) and normal rabbit IgG (●····●) were added to virus-coated wells and incubated overnight. Binding of IgG was detected by the addition of radioiodinated protein A. The levels of binding obtained have been normalised by expressing them as a percentage of the value obtained with a 1/10 dilution of anti-(X-31 virus) IgG.

The results plotted in Fig. 2 show the levels of binding obtained with 1/10 dilutions of the various IgG preparations with the different antigens. Figure 3 illustrates the types of titration curves, obtained for the binding of anti-peptide and anti-viral sera to intact virus, from which the data presented in Fig. 2 derived.

These results can be summarised as follows:

(a) antibodies raised against intact virus react strongly with virus, HA and reduced and alkylated HA, and bind slightly to the *C*-terminus HA_1 peptide;

(b) only the tip and *C*-terminus HA_1 synthetic peptides elicited antibodies which reacted with the homologous peptide;

(c) only antibodies raised against the *C*-terminus HA_1 peptide demonstrated binding to the intact virus. However, the degree of binding obtained to virus was much less than that obtained with the homologous peptide. Furthermore, this antiserum bound to intact HA but not to the reduced and alkylated form of the molecule.

In order to investigate further the specificity of antibodies elicited by the *C*-terminal HA_1 peptide, titrations were carried out against a variety of influenza virus subtypes using the solid-phase RIA. The results are presented in Fig. 4. It can be seen that antibodies directed to this synthetic peptide exhibit subtype specificity, binding occurring only to viruses of the H3 subtype. Furthermore, the binding of this anti-peptide antibody to a strain which appeared later in the H3 subtype series, A/Vic/186/82, is significantly decreased when compared with earlier H3 strains. A comparison of the amino acid sequences of the HA from the various strains (Table 2) indicates that this region is conserved within the H3 subtype but differs from the corresponding H1, H2 and H6 sequences. We have no data on the sequence of A/Vic/186/82(H3) but the binding data (Fig. 4) would indicate that there are differences between it and the earlier H3 strains examined.

Solution-phase assays

A possible explanation for the lack of binding of anti-viral antibodies to synthetic peptides in the solid-phase RIA (Fig. 2) is that binding of the peptides to polyvinyl trays may cause conformational changes in the peptides preventing their recognition by anti-viral antibodies. To avoid this, anti-viral and anti-peptide sera were tested for their binding to radiolabeled synthetic peptides in a solution-phase assay. Similar results to those observed in the solid-phase RIA were observed. The only binding observed in this assay occurred between anti-viral sera and anti-*C*-terminal peptide sera to the *C*-terminal peptide (Fig. 5). In contrast to results obtained in the solid-phase RIA, anti-tip peptide antibodies failed t bind to the tip peptide. The lack of antigenic reactivity of the tip peptide observed in the solution-phase assay may indicate that radiolabeling of this peptide destroys the epitope(s) recognised by anti-tip antibodies in the solid-phase assay. Experiments were therefore carried out in which each of the unlabeled peptides was examined for their capacity to inhibit the binding of anti-viral IgG to radioiodinated bromelain HA. No inhibition of binding was observed even when peptides were present at concns equivalent to a 3×10^{-5}-fold molar excess over HA.

It is possible that anti-(X-31) virus sera contain higher titers of antibody against a certain site(s) than against others on the HA molecule. Thus, inhibition of binding of antibody to HA by peptides derived from antigenically less important regions of the molecule may be difficult to demonstrate using polyclonal antisera. Therefore, the hinge A–B, hinge A, loop and tip peptides were examined for their ability to inhibit the binding of monoclonal antibodies H14A2, H14A20 and H14A21 to HA. These antibodies have been shown (Laver *et al.*, 1979) to select variants with amino acid substitutions in the hinge (residue 53), loop (residue 143) and tip/interface (residue 205) regions respectively. However, n significant binding of these monoclonal antibodies to the peptides could be demonstrated in direct binding solid- or solution-phase competitive RIAs.

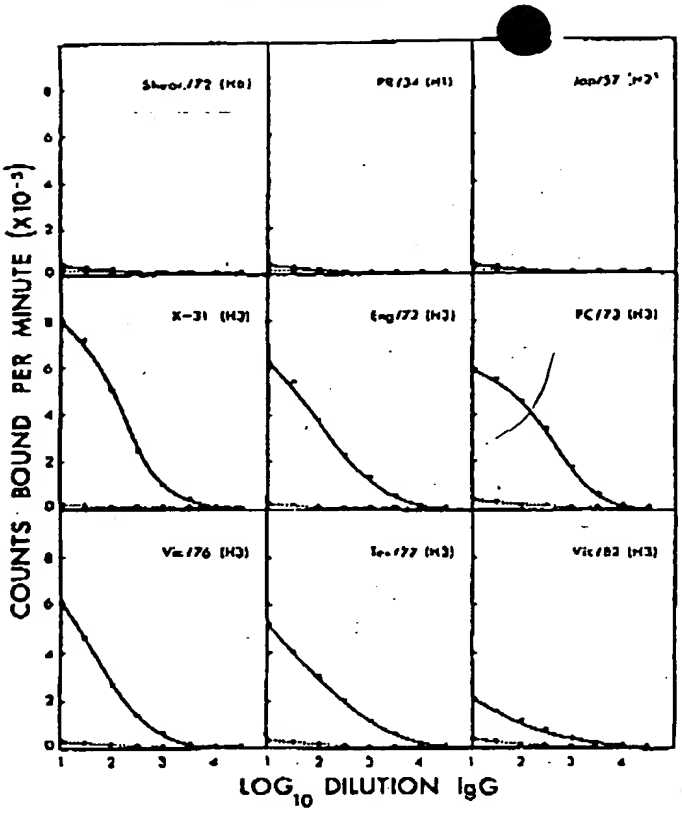

Fig. 4. Binding of anti-(HA_1 C-terminal synthetic peptide) IgG to various influenza virus subtypes and strains in solid-phase RIA. Dilutions of antibody were titrated against the different viruses and binding detected by addition of radioiodinated protein A. Anti-(HA_1 C-terminus synthetic peptide) IgG (●——●) and pre-immune IgG (O----O).

HI assays

None of the antisera raised against synthetic peptides or peptide conjugates displayed any HI activity against X-31 virus.

DISCUSSION

The synthetic peptides used in this study were homogenous as assessed by amino acid analysis and HPLC and represent authentic linear sequences within the HA of X-31 influenza virus. The loop, tip and hinge sequences were chosen as they appear (Wiley *et al.*, 1981) to comprise the major antigenic features of this protein. In order to preserve as much as possible the natural environment of the putative antigenic sequence, the free α-amino terminus of the synthetic peptides was acetylated and the carboxyl terminus was converted to the amide form. However, when examined using a variety of different assays and antisera, only one of these synthetic peptides, the HA_1 C-terminus, showed convincing cross-reactivity with purified HA or intact virus. The tip peptide when inoculated into rabbits elicited immunoglobulin which was capable of binding to the homologous peptide. No reactions were observed between these

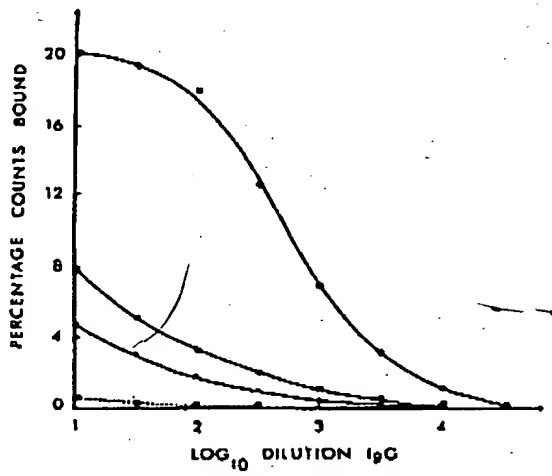

Fig. 5. Titration of antisera against radioiodinated C-terminal HA_1 peptide in solution-phase RIA. Dilutions of anti-(C-terminal HA_1) IgG (●——●), anti-(X-31 virus) IgG [(■——■) and (▲——▲)] and normal rabbit IgG (●----●) were incubated in BSA-coated centrifuge tubes with 0.3 nmoles of radioiodinated C-terminal HA_1 peptide for 3 hr at room temp. Antigen–antibody complexes were isolated by the addition of 50 μl of a 20% suspension of protein A–Sepharose CL-4B.

Table 2 Amino acid sequences of the HA, C-terminus of various influenza virus strains*

Strain	137																		160
A/PR/8/34	C	P	K	Y	V	R	S	A	K	L	R	M	V	T	G	L	R	N	I	P	S	I	Q	S
A/Jap/305/57	C	P	K	Y	V	K	S	E	K	L	V	L	A	T	G	L	R	N	V	P	Q	I	E	S
X-31	C	P	K	Y	V	K	Q	N	T	L	K	L	A	T	G	M	R	N	V	P	E	K	Q	T

* Sequences of A/Eng/42/72, A/Port Chalmers/1/73, A/Vic/11/75/76 and A/Texas/1/77 are the same as X-31.

antibodies and any of the other peptides, pure HA or intact virus. In a previous study (Jackson *et al.*, 1982) we demonstrated very low levels of binding between a synthetic loop peptide (residues 123–151) and an antiserum raised against virus. In the present study, loop peptide (residues 140–150) showed neither immunogenic nor antigenic cross-reactivity with virus.

There are a number of possible explanations for the lack of immunogenicity of the individual synthetic peptides and also their lack of antigenic cross-reactivity with intact HA.

Size

The mol. wt of an inoculated material plays an important role in its immunogenicity. A variety of native peptides such as angiotensin II (Dietrich, 1966), fibrinopeptide (Berglund, 1965) and bacitracin (Abuelo and Ovary, 1965) and peptides derived from ACTH (Salvin and Liauw, 1967), all of which have mol. wts less than 2000, are capable of eliciting antibodies. In these cases, however, the peptides were either intact or, in the case of ACTH, contained more than half of the native peptide's amino acid residues. In the present study, synthetic peptides ranging in size from nine to 24 amino acid residues (mol. wt 947–2748) were used.

Composition

Aromatic amino acid residues have been reported to enhance the amount of antibody elicited by peptides (Gill and Doty, 1961; Sela *et al.*, 1962). More recently Shi *et al.* (1984) have reported that the cationic and/or hydrophobic properties of small synthetic peptides (two–seven residues) dominate antibody-binding properties and conclude that antibody binding to such small peptides cannot be interpreted in terms of biologically relevant antigenic specificity. Lerner *et al.* (1983) investigating the immunogenic properties of seven synthetic peptides representing portions of the sequence of hepatitis B surface antigen found that the four peptides which did not elicit antibody production were hydrophobic in nature. Of the peptides examined here, however, only the fusion peptide is hydrophobic and it is therefore difficult to make any generalisations from our data about the hydrophobic or hydrophilic requirements necessary for immunogenic activity.

Conformation

Many of the antigenic determinants described to date possess secondary- and tertiary-order structures which are brought into juxtaposition by folding of the polypeptide chain (Arnon and Sela, 1969; Arnon, 1971; Crumpton, 1974). Evidence suggests that peptides shorter than 30 amino acid residues do not adopt stable configurations in solution (Sheraga, 1981). It is believed that they exist in a large number of different and transient conformational states which are in equilibrium. At any particular time, that proportion of peptide molecules existing in the conformation most representative of the native configuration may be as little as 10^{-4}–10^{-5}. For this reason, an inoculum of synthetic peptide may be thought of as containing many thousands of conformationally and hence antigenically distinct species. The equilibrium constant for antibody–antigen interaction between synthetic peptides and antibodies raised against the intact parent protein or between anti-peptide antibodies and native antigen may therefore be reduced by a factor of 10^4–10^5 (Sachs, 1974).

Despite the apparent theoretical limitations of the synthetic peptide approach many groups have reported raising antisera to synthetic peptides and shown that these react with native proteins (Langbeheim *et al.*, 1976; Sutcliffe *et al.*, 1980; Lerner *et al.*, 1981; Green *et al.*, 1982; Lerner, 1982). These findings indicate that: (a) conformations of the peptides which mimic the native conformation are present in the inoculum in sufficient concn to elicit specific antibodies which will recognise the native antigen, (b) the antigenic determinants which the anti-peptide antibodies recognise on the native antigen are merely strings of amino acid residues possessing no conformational information, or (c) antibodies elicited by the synthetic peptides are able to induce some conformational change in the native protein enabling antibody–antigen complex formation.

With the exception of the C-terminus peptide we have not been able to demonstrate binding between anti-peptide antibodies and native antigen or between anti-viral antibodies and synthetic peptides. In fact not all of our peptides elicited antibody production in rabbits even when coupled to carriers. One explanation of these results may be that the antigenic regions of HA proposed by Wiley *et al.* (1981) do not represent major antigenic features of the HA molecule. Evidence that these regions comprise antigenic determinants is indirect, based on sequence studies carried out on variant HA molecules selected in the presence of monoclonal antibodies (Laver *et al.*,

1979) and a comparison of the amino acid sequences of field strain isolates (Wiley *et al.*, 1981).

It is, however, more likely that the loop, tip and hinge regions are sites of antigenic activity within the native molecule and that the antigenicity of these sites is dependent on conformation. It is possible that insufficient information is incorporated into the synthetic peptides we have assembled to represent these sites. These peptides are thus unable to assume the correct conformation represented in the native antigen. HA possesses five disulphide bridges which are necessary for the molecule's structural and functional integrity. In a protein where such a large number of covalent bridges are required to constrain the linear sequence of amino acid residues into its final conformation, it is likely that the antigenic sites are comprised of structural elements which may be remote in sequence but brought into juxtaposition as the intact molecule folds into its final conformation. Inspection of the three-dimensional antigenic map of HA (Wiley *et al.*, 1981) supports this contention where it can be seen that the loop, tip and hinge regions possess definite conformational structures.

It is likely that our synthetic peptides exist as "pieces of string" in aq. phase, i.e. they do not contain the inherent structural features of the native molecule which would allow them to mimic immunogenically and antigenically the sequences they represent. Perhaps this is not surprising when considered in the light of experiments where low immunogenic and antigenic activities were obtained using much larger fragments of HA (Jackson *et al.*, 1979*a*, *b*).

The results reported here contrast with those of Green *et al.* (1982) who attach less significance to conformational requirements. They report that most of their synthetic peptides representing some 75% of the HA_1 sequence from X47 influenza HA, including very short sequences not present at the molecule's surface, elicit antibodies which bind to HA. Neither could we obtain antibodies using the *N*-terminal portion of HA_2 as an immunogen, a peptide which has been reported (Atassi and Webster, 1983) to be capable of binding anti-viral antibody and also of inducing antibodies which bind to intact virus. Shapira *et al.* (1984) have studied in more detail the immunogenic and antigenic features of the loop region. Their findings show that antibodies raised against a synthetic loop peptide (residues 139–146) do not recognise intact virus but that longer peptides (residues 138–164 and 147–164), which include regions additional to the loop, do induce antibodies which react with virus.

The present studies may be taken as a cautionary note amidst the numerous reports on the use of synthetic peptides which have recently appeared in the literature. It seems that in some cases the size, and ultimately the conformation, of synthetic peptides will govern their immunogenic and antigenic properties—a possibility already supported by one report (Ionescu-Matiu *et al.*, 1983).

REFERENCES

Abuelo J. G. and Ovary Z. (1965) Dinitrophenylated bacitracin as an antigen in the guinea pig. *J. Immun.* 95, 113–117.

Altschuh D., Hartman D., Reinbolt J. and Van Regenmortel M. H. V. (1983) Immunochemical studies of tobacco mosaic virus—V. Localization of four epitopes in the protein subunit by inhibition tests with synthetic peptides and cleavage peptides from three strains. *Molec. Immun.* 20, 271–278.

Arnon R. (1971) Antibodies to enzymes—a tool in the study of antigenic specificity determinants. *Curr. Topics Microbiol. Immun.* 54, 47–94.

Arnon R. and Sela M. (1969) Antibodies to a unique region in lysozyme provoked by a synthetic antigen conjugate. *Proc. natn. Acad. Sci. U.S.A.* 62, 163–170.

Atassi M. Z. and Webster R. G. (1983) Localization, synthesis and activity of an antigenic site on influenza virus hemagglutinin. *Proc. natn. Acad. Sci. U.S.A.* 80, 840–844.

Barany G. and Merrifield R. B. (1980) In *The Peptides, Analysis, Synthesis, Biology* (Edited by Gross E. and Meienhofer J.), Vol. 2, pp. 1–255. Academic Press, New York.

Baron M. H. and Baltimore D. (1982) Antibodies against a synthetic peptide of the poliovirus replicase protein: reaction with native virus encoded proteins and inhibition of virus-specific polymerase activities *in vitro*. *J. Virol.* 43, 969–978.

Berglund G. (1965) Preparation of antiserum to an antigen of low molecular weight. *Nature, Lond.* 206, 523–524.

Bittle J. L., Houghten R. A., Alexander H., Shinnick T. M., Sutcliffe J. G., Lerner R. A., Rowlands D. J. and Brown F. (1982) Protection against foot and mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. *Nature, Lond.* 298, 30–33.

Bolton A. E. and Hunter W. H. (1973) The labeling of proteins to high specific radioactivity by conjugation to a ^{125}I-containing acylating agent. Application to the radioimmunoassay. *Biochem. J.* 133, 529–538.

Brand C. M. and Skehel J. J. (1972) Crystalline antigen from the influenza virus envelope. *Nature New Biol.* 238, 145–147.

Breschkin A. M., Ahern J. and White D. O. (1981) Antigenic determinants of influenza virus hemagglutinin VIII. Topography of the antigenic regions of influenza virus hemagglutinin determined by competitive radioimmunoassay with monoclonal antibodies. *Virology* 113, 130–140.

Brown L. E., Dopheide T. A. A., Ward C. W., White D. O. and Jackson D. C. (1980) Antigenic determinants of influenza virus hemagglutinin V. Antigenicity of the HA_2 chain. *J. Immun.* 125, 1583–1588.

Crumpton M. J. (1974) Protein antigens: the molecular bases of antigenicity and immunogenicity. In *The Antigens* (Edited by Sela M.), Vol. 2, pp. 1–72. Academic Press, New York.

Dietrich F. M. (1966) Immunogenicity of synthetic angiotensin II. *Int. Archs Allergy appl. Immun.* 30, 497–506.

Emini E. A., Jameson B. A. and Wimmer E. (1983) Priming for and induction of anti-poliovirus neutralizing antibodies by synthetic peptides. *Nature, Lond.* 304, 699–703.

Fazekas de St. Groth S. and Webster R. G. (1966) Disquisitions on original antigenic sin I. Evidence in man. *J. exp. Med.* 124, 331–345.

Gerhard W., Yewdell J., Frankel M. E. and Webster R. G. (1981) Antigenic structure of influenza virus hemagglutinin defined by hybridoma antibodies. *Nature, Lond.* 290, 713–717.

Gething M. J., White J. M. and Waterfield M. D. (1978) Purification of the fusion protein of Sendai virus: analysis

of the NH_2-terminal sequence generated during precursor activation. *Proc. natn. Acad. Sci. U.S.A.* 75, 2737–2740.

Gill T. J. III and Doty P. (1961) Studies on synthetic polypeptide antigens II. The immunochemical properties of a group of linear synthetic polypeptides. *J. biol. Chem.* 236, 2677–2683.

Goodfriend T. L., Levine L. and Fasman G. D. (1964) Antibodies to bradykinin and angiotensin: a use of carbodiimides in immunology. *Science* 144, 1344–1346.

Grandgenett D. P., Knaus R. J. and Hippenmeyer P. J. (1983) Antibodies against a synthetic peptide of the avian retrovirus pp32 protein and the β DNA polymerase subunit. *Virology* 130, 257–262.

Green N., Alexander H., Olson A., Alexander S., Shinnick T. M., Sutcliffe J. G. and Lerner R. A. (1982) Immunogenic structure of the influenza virus hemagglutinin. *Cell* 28, 477–487.

Greenwood F. C., Hunter W. M. and Glover J. S. (1963) The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* 89, 114–123.

Huang R. T. C., Rott R. and Klenk H. D. (1981) Influenza viruses cause hemolysis and fusion of cells. *Virology* 110, 243–247.

Ionescu-Matiu I., Kennedy R. C., Sparrow J. T., Culwell A. R., Sanchez Y., Melnick J. L. and Dreesman G. R. (1983) Epitopes associated with a synthetic hepatitis B surface antigen peptide. *J. Immun.* 130, 1947–1952.

Jackson D. C. (1980) Some effects of chloramine-T induced radioiodination on the physico-chemical properties of oligomeric proteins. *J. immun. Meth.* 34, 253–260.

Jackson D. C., Brown L. E. and White D. O. (1981) Antigenic determinants of influenza virus hemagglutinin. VI. Antigenic characterisation of the oligosaccharide side chains from HA_1 of influenza virus hemagglutinins. *J. gen. Virol.* 52, 163–168.

Jackson D. C., Brown L. E., White D. O., Dopheide T. A. A. and Ward C. W. (1979*a*) Antigenic determinants of influenza virus hemagglutinin. IV. Immunogenicity of fragments isolated from the hemagglutinin of A/Memphis/72. *J. Immun.* 123, 2610–2617.

Jackson D. C., Dopheide T. A., Russell R. J., White D. O. and Ward C. W. (1979*b*) Antigenic determinants of influenza virus hemagglutinin. II. Antigenic reactivity of the isolated *N*-terminal cyanogen bromide peptide of A/Memphis/72 hemagglutinin heavy chain. *Virology* 93, 458–465.

Jackson D. C., Murray J. M., White D. O., Fagan C. N. and Tregear G. W. (1982*a*) Antigenic activity of a synthetic peptide comprising the "loop" region of influenza virus hemagglutinin. *Virology* 120, 273–276.

Jackson D. C., Murray J. M., White D. O. and Gerhard W. U. (1982*b*) Enumeration of antigenic sites of influenza virus hemagglutinin. *Infect. Immun.* 37, 912–918.

Jackson D. C., Russell R. J., Ward C. W. and Dopheide T. A. (1978) Antigenic determinants of influenza virus hemagglutinin. I. Cyanogen bromide peptides derived from A/Memphis/72 hemagglutinin possess antigenic activity. *Virology* 89, 199–205.

Kent S. B. H. (1980) In *Biomedical Polymers* (Edited by Goldberg E. P. and Nakajima A), pp. 213–242. Academic Press, New York.

Langbeheim H., Arnon R. and Sela M. (1976) Antiviral effect on MS-2 coliphage obtained with a synthetic antigen. *Proc. natn. Acad. Sci. U.S.A.* 73, 4636–4640.

Laver W. G. (1964) Structural studies on the protein subunits from three strains of influenza virus. *J. molec. Biol.* 9, 109–124.

Laver W. G. (1971) Separation of two polypeptide chains from the hemagglutinin subunit of influenza virus. *Virology* 45, 275–288.

Laver W. G., Air G. M., Webster R. G., Gerhard W., Ward C. W. and Dopheide T. A. (1979) Antigenic drift in type A influenza virus: sequence differences in the hemagglutinin of Hong Kong (H3N2) variants selected with monoclonal hybridoma antibodies. *Virology* 98, 226–237.

Lerner R. A., Green N., Alexander H., Liu F.-T., Sutcliffe J. G. and Shinnick T. M. (1981) Chemically synthesized *Nature, Lond.* 299, 592–596

Lerner R. A., Green N., Alexander H., Liu F.-T., Sutcliffe J. G. and Shinnick T. M. (1981) Chemically synthesized peptides predicted from the nucleotide sequence of the hepatitis B virus genome elicit antibodies reactive with the native envelope protein of Dane particles. *Proc. natn. Acad. Sci. U.S.A.* 78, 3403–3407.

Müller G. M., Shapira M. and Arnon R. (1982) Anti-influenza response achieved by immunisation with a synthetic conjugate. *Proc. natn. Acad. Sci. U.S.A.* 79, 569–573.

Prince A. M., Ikram H. and Hopp T. P. (1982) Hepatitis B virus vaccine: identification of HBsAg/a and HBsAg/d but not HBsAg/y subtype antigenic determinants on a synthetic immunogenic peptide. *Proc. natn. Acad. Sci. U.S.A.* 79, 579–582.

Quesniaux U., Briand J.-P. and Van Regenmortel M. H. V. (1983) Immunochemical studies of turnip yellow mosaic virus—II. Localization of a viral epitope in the *N*-terminal residues of the coat protein. *Molec. Immun.* 20, 179–185.

Richardson C. D., Scheid A. and Choppin P. W. (1980) Specific inhibition of paramyxovirus replication by oligopeptides with amino acid sequences similar to those at the *N*-termini of the F_1 or HA_2 viral polypeptides. *Virology* 105, 205–222.

Rowlands D. J., Clarke B. E., Carroll A. R., Brown F., Nicholson B. H., Bittle J. L., Houghten R. A. and Lerner R. A. (1983) Chemical basis of antigenic variation in foot-and-mouth disease virus. *Nature, Lond.* 306, 694–697.

Sachs D. H. (1974) In *Current Topics in Biochemistry* (Edited by Anfinson C. B. and Schechter A. N.). Academic Press, New York.

Salvin S. B. and Liauw H.-L. (1967) Hypersensitivity to peptide fragments. *Int. Archs Allergy appl. Immun.* 31, 366–379.

Sela M., Fuchs S. and Arnon R. (1962) Studies on the chemical basis of the antigenicity of proteins. 5. Synthesis, characterisation and immunogenicity of some multichain and linear polypeptides containing typrosine. *Biochem. J.* 85, 223–235.

Shapira M., Jibson M., Muller G. and Arnon R. (1984) Immunity and protection against influenza virus by synthetic peptide corresponding to antigenic sites of hemagglutinin. *Proc. natn. Acad. Sci. U.S.A.* 81, 2461–2465.

Sheraga H. A. (1981) Influence of interatomic interactions on the structure and stability of polypeptides and proteins. *Biopolymers* 20, 1877–1899.

Shi., Riehm J. P., Todd P. E. E. and Leach S. J. (1984) The antigencity of myoglobin-related peptides synthesized on polyacrylamide and polystyrene resin supports. *Molec. Immun.* 21, 489–496.

Skehel J. J., Bayley P. M., Brown E. B., Martin S. R., Waterfield M. D., White J. M., Wilson I. A. and Wiley D. C. (1982) Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc. natn. Acad. Sci. U.S.A.* 79, 968–972.

Skehel J. J. and Waterfield M. D. (1975) Studies on the primary structure of the influenza virus hemagglutinin. *Proc. natn. Acad. Sci. U.S.A.* 72, 93–97.

Sutcliffe J. G., Shinnick T. M., Green N., Liu F.-T., Nimun H. L. and Lerner R. A. (1980) Chemical synthesis of a polypeptide predicted from nucleotide sequence allows detection of a new retroviral gene product. *Nature, Lond.* 287, 801–805.

Tamura T., Bauer H., Birr C. and Pipkorn R. (1983)

Antibodies against synthetic peptides as a tool for functional analysis of the transforming protein pp60^{v-src}. *Cell* 34, 587–596.

Ward C. W. (1981) Structure of the influenza hemagglutinin. *Curr. Topics Microbiol.* 94/95, 1–74.

Webster R. G. and Laver W. G. (1980) Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. *Virology* 104, 139–148.

White J. M., Matlin K. and Helenius A. (1981) Cell fusion by Semliki forest, influenza and vesicular stomatitis viruses. *J. Cell Biol.* 89, 674–679.

Wiley D. C., Wilson I. A. and Skehel J. J. (1981) Structural identification of the antibody-binding sites of Hong Kong influenza hemagglutinin and their involvement in antigenic variation. *Nature, Lond.* 289, 373–378.

Wilson I. A., Wiley D. C. and Skehel J. J. (1981) Structure of the hemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature, Lond.* 289, 366–373.